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ENHANCEMENT OF ANTIBODY PRODUCTION BY ANTI-DELTA
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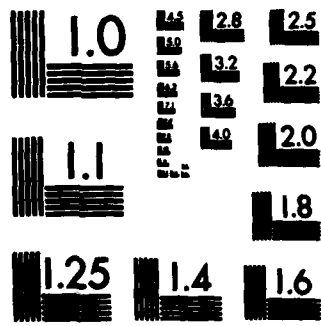
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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) The abilities of four stimuli to activate B lymphocytes <u>in vivo</u> and in- duce B cells to proliferate and differentiate into antibody secreting cells were studied. A goat antibody to mouse IgD (GaM δ) stimulates T independent increases in B cell DNA synthesis and T dependent increases in spleen cell number and IgG ₁ secretion. Serum IgG ₁ levels increase to 50-100 times con- trol levels, and 5-15% of the Ig secreted is goat IgG specific. Autoantibody		

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production is not detectable. Injection of GaM6 plus a toxin - normal goat IgG conjugate stimulates rapid production of large quantities of IgG, anti-toxin antibody, and facilitates the generation of anti-toxin secreting monoclonal antibodies. A second agent, 8-mercaptoguanosine (8MG) stimulates neither B cell proliferation nor antibody production by itself, but enhances antigen specific antibody responses, particularly IgG, responses. A third agent, lipid A, stimulates B cell proliferation but fails in vivo to increase serum IgM or IgG levels. Combinations of GaM6, lipid A, and 8MG synergistically induce large increases in spleen cell number and polyclonal IgG, secretion but fail to stimulate a polyclonal IgG, response or a specific anti-goat IgG response. A fourth stimulus, a non-lethal graft vs. host reaction (GVH) induces large increases in serum IgM and IgG levels, which include autoantibody production. No synergy was seen in the abilities of GaM6 and GVH to stimulate increases in spleen cell number or serum Ig levels. These observations help define alternative pathways of B cell activation and provide procedures for the rapid generation of polyclonal and monoclonal anti-toxin antibodies.



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Annual Report - Enhance of Antibody Production by Anti- δ Antibodies

Four strategies for the promotion of *in vivo* B lymphocyte activation have been studied with the initial aims of 1) determining whether polyclonal B cell activation would result in the production of sufficient levels of Ig specific for toxic or bacterial antigens to protect against these agents; 2) determining whether the same stimuli could enhance production of antibodies to toxins or bacterial antigens; and 3) elucidation of the mechanisms involved in polyclonal B cell activation in the system studied. During the course of this contract an additional aim has been added: rapid production of monoclonal antibodies to toxin or bacterial products.

The four strategies studied were: 1) injection of mice with affinity purified heterologous antibodies to mouse IgD, particularly goat anti-mouse IgD (GaM δ), 2) injection of mice with 8-mercaptoguanosine (8 MG), 3) injection of mice with lipid A derived from *S. Minnesota* LPS (purchased from Ribi), and 4) generation of a stimulatory graft vs. host reaction. In addition to studying the effects of single stimuli I have studied the effects of combinations of these stimuli on B cell activation.

a) Studies with GaM δ : Injection of BALB/c mice with 200-800 μ g of GaM δ has a number of activating effects on B lymphocytes that are seen 24-48 hours after i.v. injection and are T independent. These include: a) enhanced expression of Ia antigen, enhanced ability to present antigen to T lymphocytes, enhanced expression of transferrin receptor and Con A TRF receptor, and loss of the homing receptor (Mel 14); b) induction of increases in size of approximately 80% of splenic B cells; and c) induction of DNA synthesis by 25-50% of B cells. Stimulation of T cell DNA synthesis, which is dependent upon antigen presentation by GaM δ activated B cells to goat IgG specific T cells, is seen 2-3 days after GaM δ injection. In the presence of goat IgG specific T lymphocytes B cells are stimulated to differentiate into IgG₁ secreting cells and to proliferate. By 7-8 days after GaM δ injection a 3-8 fold increase in spleen cell number is seen and up to 50% of splenic B cells secrete IgG₁. Nine days after GaM δ injection serum IgG₁ levels have increased to 30-60 mg/ml (50-100 times baseline values) and 5-20% of the IgG₁ secreted is specific for goat IgG. Autoantibody responses, if they occur at all, are probably minimal, since IgG₁ anti-mouse erythrocyte antibodies and anti-nuclear antibodies are undetectable. Antibodies to foreign toxins or bacterial antibodies are also undetectable. This is probably because those B cell clones specific for such antigens represent such a small fraction of the B cell repertoire that even a 100 fold increase in the level of antibodies to such antigens would not be detected. Injection of foreign antigens, including toxins, along with GaM δ , often increases the quantity of antibody produced to these antigens. This probably reflects increased antigen presentation by GaM δ activated B cells. Much greater enhancement of antibody production to specific antigens (often more than 1,000 fold) is achieved by injecting GaM δ along with antigen conjugated to normal goat IgG. This observation is useful for the production of antibodies to small toxins, such as the trichothecine toxin T₂, which can be treated as haptens and conjugated to goat IgG. Optimal anti-toxin production is achieved when 1-2 molecules of toxin are conjugated to each goat IgG molecule, and mice are injected with 200 μ g of GaM δ plus 400 μ g of toxin-goat IgG conjugate but doses of toxin-goat IgG conjugate, as low as 1 μ g still induce substantial anti-toxin antibody production. Considerable IgG₁ anti-toxin antibody production is seen 7 days after mice are immunized. In collaboration with Dr. Kenneth Hunter I have studied the use of this system for the generation of hybridomas that produce

specific antibodies. When fusions are performed 5-8 days after GaM δ plus hapten-goat IgG conjugates injection large numbers of hybridomas that secrete specific anti-hapten antibody are produced. While fluorescein isothiocyanate (FITC) has been the hapten used for most of these studies, this technique has been used to make 2 monoclonal antibodies to the trichothecene toxin T₂. In studies with GaM δ plus T₂-goat IgG, however, hybrid cell growth following fusion has been poor. Minor modifications of the immunization and fusion process have corrected this problem, and we hope soon to be able to use the GaM δ system to produce large numbers of hybrids specific for T₂ and other small toxins.

Studies were also performed to determine the most effective way of boosting primary antibody responses produced with the GaM δ system. FITC-Ficoll, FITC-Brucella abortus (FITC-BA), FITC-sheep erythrocytes, FITC-LPS, FITC-heterologous spleen cells; FITC-Staphylococcus aureus, FITC-B. pertussis, rabbit anti-mouse δ (RaM δ) plus FITC-rabbit IgG, or saline were injected into mice that had been primed 14 days earlier with GaM δ plus FITC-goat IgG. IgG₁ anti-FITC titers were obtained for sera drawn 10 days after the primary immunization or 9 days after the boost. Surprisingly, titers obtained 10 days after the primary immunization were highest. In the absence of a boost titers declined precipitously. Anti-FITC titers of mice boosted with FITC-BA or RaM δ plus FITC-rabbit IgG were considerably larger than those seen in mice boosted with saline or with the other FITC-antigens used. Relative avidities of anti-FITC antibodies were determined by an ELISA adaptation of the microtiter plate RIA used by Herzenberg. Six FITC-bovine serum albumin (BSA) conjugates with molar FITC-BSA conjugation ratios ranging from 0.5 to 20 were bound to microtiter plate wells and sera were titered for anti-FITC activity on microtiter plates coated with each of these conjugates. Low avidity antibodies bind much better to plates coated with highly conjugated FITC-BSA; higher avidity anti-FITC antibodies will also bind to plates coated with less heavily conjugated FITC-BSA. Thus, the avidity of anti-FITC antibodies produced will be proportional in a general way to the titer obtained on plates coated with lightly conjugated FITC-BSA, divided by the titer obtained on plates coated with heavily conjugated FITC-BSA. Sera from mice initially immunized with GaM δ plus FITC-goat IgG, and then boosted with either FITC-BA or RaM δ plus FITC-rabbit IgG had lower titers of IgG₁ anti-FITC than did day 10 sera from mice given only the primary immunization when analyzed on FITC₂₀ BSA coated plates, but much higher titers of IgG₁ anti-FITC when analyzed on FITC_{0.5} BSA plates. Thus boosting considerably increased antibody avidity. Serum antibodies obtained from mice ten days after "boosting" with saline, unlike antibodies from mice boosted with FITC-BA, were not increased in apparent affinity when compared to antibodies in sera obtained 10 days after the primary immunization. This microtiter technique, while it provides affinity data less precise than is available by equilibrium dialysis or the Farr technique, is particularly valuable for its speed, its ability to analyze very small quantities of antibody, and its ability to selectively examine avidity of a single antibody isotype. It is particularly well suited for the early characterization of monoclonal antibodies, since it can be used to select for high affinity antibody producing clones before tedious cloning and clonal expansion procedures are performed.

b) Studies with 8-mercaptoinosine (8MG). Goodman and colleagues have demonstrated that the purine derivative 8MG enhances in vivo antibody responses and acts as a polyclonal mitogen and inducer of antibody secretion in vitro. We have studied the in vivo ability of 8MG to directly activate B cells and to enhance specific and polyclonal antibody responses to GaM δ , normal goat IgG, sheep erythrocytes, and TNP-Ficoll in normal and nude mice. In most experiments

mice were injected i.p. with 30 mg of 8MG suspended in 0.2 ml of 2% carboxymethylcellulose (CMC) at the same time as they were injected with antigen. 8MG was found to have only very slight direct B cell activating effects. Small increases in B cell s1a expression and size were seen 7 days after 8MG injection; no increases in splenic B cell number were observed. When injected with normal goat IgG both the IgG₁ and IgG₂ anti-goat IgG responses were increased considerably above those seen in mice injected with goat IgG plus CMC although the IgG₂ response was increased to a greater extent than the IgG₁ response. 8MG appeared to slightly decrease the IgG₁ polyclonal and goat IgG-specific antibody responses noted in mice injected with GaM6, but substantially increased the IgG₂ polyclonal and goat IgG-specific responses in these mice. 8MG did not affect the IgG₁ anti-sheep erythrocyte (SRBC) response but did increase the IgG₂ anti-SRBC response in SRBC immunized mice. Administration of 8MG during a primary immunization with SRBC substantially increased both the primary and secondary IgG₂ anti-SRBC responses in mice boosted 14 days after priming, while administration of 8MG at the time of boosting did not affect the size of the IgG₁ or IgG₂ secondary response, regardless of whether 8MG was given with the primary immunization. Thus, 8MG can enhance memory B cell production, but does not seem to enhance memory B cell activation. 8MG, when injected with TNP-Ficoll, enhanced IgM, IgG₁, and IgG₂ anti-TNP responses, but the IgG₂ response was enhanced to the greatest extent. Nude mice injected with 8MG did not generate antibody responses to SRBC, goat IgG, or GaM6, but did show enhanced antibody responses, especially enhanced IgG₂ responses, to TNP-Ficoll. Thus, 8MG fails to substitute completely for T cell help in nude mice, but can stimulate antibody production by the B cells found in these mice when they receive the additional stimuli that are provided by TNP-Ficoll, a relatively T independent antigen. While GaM6 plus a single dose of 8MG failed to enhance spleen cell number in nude mice, nude mice injected initially with GaM6 plus 30 mg of 8MG that received additional 30mg doses of 8MG 1 and 2 days later showed a 5 fold increase in spleen cell number. No polyclonal IgG₁ or IgG₂ antibody production or any goat IgG specific antibody production was seen. However a several-fold polyclonal increase in serum IgG₂ levels was induced. Injection of nude mice with three 30mg doses of 8MG but no GaM6 failed to induce either an increase in spleen cell number or an increase in serum Ig levels. Thus, 8MG appears to have a short half life *in vivo*. When injected in multiple doses 8MG can promote growth as well as differentiation of GaM6 activated B cells to IgG₂ secreting cells, but fails to promote growth of resting B cells. For B cells stimulated to secrete Ig of any isotype by T dependent or relatively T independent antigens, 8MG can enhance antibody production, particularly IgG₂ antibody production. Practical uses of 8MG and similar compounds should include enhancement of IgG responses to carbohydrate antigens and stimulation of generation of hybridomas that secrete IgG₂ antibodies, which are much easier to purify than IgG₁ antibodies.

c) Experiments with Lipid A. To my surprise, lipid A, in doses up to 50 µg/mouse, fails to increase serum IgG or IgM levels in normal or nude mice, although it stimulates B cell DNA synthesis and induces several-fold increases in the number of splenic B cells and splenic non-B, non-T cells in both normal and nude mice. Lipid A, injected into normal mice with GaM6, fails to increase the polyclonal or anti-goat IgG responses generated in these mice. When injected into normal mice with normal goat IgG, lipid A greatly enhances the specific IgG₁ anti-goat IgG response (to approximately 25% that seen in GaM6 injected mice) but unlike GaM6 fails to appreciably enhance total serum IgG₁ levels. When injected into nude mice with GaM6 and three consecutive daily

doses of 8MG, lipid A increases spleen cell number by a factor of 4 above that seen with GaM6 plus 8MG alone (20 fold above unstimulated values) but fails to induce specific anti-goat IgG antibody production or a polyclonal IgG₁ antibody response. The polyclonal IgG_{2a} response induced by GaM6 plus multiple doses of 8MG is, however, enhanced by lipid A. Thus, lipid A by itself has a mitogenic effect in vivo, which can act synergistically with other mitogenic stimuli, and, like 8MG, can enhance antibody production induced by poor antigens, but cannot totally replace T help or other agents that are capable of stimulating B cells to secrete antigen.

d) Experiments with a stimulatory graft vs. host reaction (GVH). Gleichmann and colleagues have reported that injection of (DBA/2 x C57BL/6)F₁ mice with C57BL/6 T cells induces a stimulatory GVH in which serum Ig levels increase appreciably. It was noted that B cells specific for autoantibodies were stimulated to produce IgG antibody to a greater extent in this system than were B cells specific for foreign antigens. This led Gleichmann to hypothesize that B cell sIg crosslinking was the limiting factor in B cell activation in this system, and led me to study if GaM6 and a stimulatory GVH would act synergistically to stimulate polyclonal B cell activation greater than that induced by either stimulus alone. The small number of experiments performed so far indicate that this is probably not the case. GaM6 induces a more rapid polyclonal antibody response in GVH mice than in normal mice, but the peak antibody response is no greater. This may be due to induction by GaM6 of T cell suppression that limits the size of the response seen. In support of this hypothesis is the observation that GVH mice injected with GaM6 appear to develop a considerably lower anti-nuclear antibody response than that seen in GVH mice that do not receive GaM6.

Future Directions

Studies to be performed in the next (and anticipated last) year of this contract will concentrate on three areas.

1) Attempts will be made to find synergistic combinations of stimuli in nude mice that will induce polyclonal Ig secretion of the magnitude seen in GaM6 injected mice. To the stimuli (GaM6, lipid A, and multiple doses of 8MG) already shown to induce polyclonal B cell proliferation will be added supernatants of activated T lymphocytes (Con A stimulated normal T cells, PMA stimulated EL4 cells, etc.). Since TNP-Ficoll stimulates anti-TNP antibody responses in nude mice, GaM6-Ficoll conjugates are being prepared and will be analyzed for their ability to trigger polyclonal Ig secretion in nude mice.

2) I will try to find combinations of stimuli that will polyclonally activate B cells in normal mice to the same extent as are goat IgG-specific B cells in GaM6 injected mice. Further experiments will be carried out in the GVH system, especially in GVH mice in which suppressor T cells have been eliminated with an anti-Lyb2 antibody. Lipid A and bacterial proteins that have polyclonal activator activity in vitro will be directly conjugated to GaM6 and injected in vivo. Further studies will be performed in which mice receive multiple doses of 8MG in an attempt to stimulate maximal polyclonal expansion. To eliminate the effects of T cell suppression some of these studies will be performed in BALB/c nude mice that have been reconstituted with anti-Lyb2 plus complement treated BALB/c splenic T cells.

3) I will optimize conditions for the rapid generation of high affinity monoclonal antibodies to toxins and bacterial products. The four stimuli described in this report will be analyzed for their abilities to generate specific monoclonal antibodies. Emphasis will be put on using these agents together to generate IgG₂ antibodies. Optimal boosting procedures, antigen and adjuvant doses, time between immunization and fusion, and early detection of high avidity antibodies with the microtiter plate assay described above will be studied. I am applying to agencies of DOD other than ONR for funds to carry out this third aim, and will remove this aim from projected work on my ONR contract if the outside contracts are awarded.

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Patents:

1. Finkelman, F.D. Enhancement of specific antibody production with anti-IgD antibodies. Disclosure of invention submitted to ONR, 27 July 1983.

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